

## Endothelin Receptor Antagonists Inhibit Endothelin in Human Skin Microcirculation

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**Abstract** Endothelin is a potent vasoconstrictor peptide produced by endothelial cells, but its role in physiology and disease is uncertain. We investigated the influence of the endothelin-A-selective receptor antagonist PD 147953 and the nonselective endothelin receptor antagonist PD 145065 on the effects of endothelin-1 and endothelin-3 in the skin microcirculation of healthy volunteers, using laser Doppler flowmetry. A double injection model was developed, allowing simultaneous injection of two substances, ie, agonist and antagonist or saline. The injection of saline led to a well-defined vasodilation at the injection site (maximum increase, from  $19 \pm 2$  to  $97 \pm 15$  perfusion units at 6 minutes;  $P < .001$ ;  $n = 10$ ). Endothelin-1 ( $10^{-12}$  mol) decreased blood flow (difference from saline control,  $-79 \pm 14$  perfusion units;  $P < .001$ ;  $n = 11$ ) within the injection wheal (diameter, 4 to 5 mm), while endothelin-3 had no effect. In the surrounding area (at 8 mm from the injection site), both endothelin-1 ( $+116 \pm 32$  perfusion units;  $P < .001$ ;  $n = 11$ ) and endothelin-3 ( $+59 \pm 16$  perfusion units;  $P < .001$ ;  $n = 11$ ) markedly increased blood flow. Both endothelin receptor antagonists slightly increased blood flow (maximum differ-

ence from control,  $+56 \pm 18$  [PD 147953] and  $+31 \pm 10$  [PD 145065] perfusion units;  $P < .05$ ;  $n = 8$ ) and inhibited endothelin-1-induced ( $P < .01$ ) vasoconstriction. The vasoconstriction to norepinephrine was not affected by the endothelin antagonist PD 147953. Endothelin-1- and endothelin-3-induced vasodilation in the surrounding area were also inhibited by both endothelin antagonists or by lidocaine. Thus, in human skin microcirculation endothelin-1 but not endothelin-3 induced pronounced vasoconstriction at the injection site and neurally mediated vasodilation in the surrounding area. PD 147953, an endothelin-A receptor antagonist, and PD 145065, a nonselective endothelin antagonist, caused slight vasodilation and prevented vasoconstriction to endothelin-1 and the neurogenic vasodilation to endothelin-1 and endothelin-3. This suggests that in human skin microcirculation endothelin-1 is involved in the regulation of vascular tone primarily by activation of endothelin-A receptors. (*Hypertension*. 1994;23:581-586.)

**Key Words** • endothelins • laser-Doppler flowmetry • lidocaine • norepinephrine • microcirculation

Endothelium-derived factors such as nitric oxide, prostacyclin,<sup>1</sup> and endothelin-1<sup>2,3</sup> participate in the regulation of vascular tone. The receptors activated by endothelins are of the endothelin-A (ETA) or endothelin-B (ETB) type.<sup>4-10</sup> In smooth muscle cells both receptors can mediate vasoconstriction, while ETB receptors on endothelial cells cause vasodilation through the release of nitric oxide and prostacyclin.<sup>11,12</sup> ETA receptors have a high affinity to endothelin-1 and a lesser affinity to endothelin-3, whereas ETB receptors bind endothelin-1 and endothelin-3 equally well.<sup>4,5,13</sup>

The role of endothelins in physiology and disease is unclear. Infusion of endothelin leads to transient vasodilation followed by long-lasting and profound vasoconstriction.<sup>2,11,12,14</sup> The role of endogenous endothelin in humans is unknown, but animal studies suggest that it may contribute to blood pressure regulation.<sup>15</sup> Similarly, although the role of endothelin in arterial hypertension is controversial,<sup>15,16</sup> it may be involved in the development and/or maintenance of high blood pressure and its complications such as arteriosclerosis,<sup>17</sup> myocardial infarction,<sup>18,19</sup> and stroke.<sup>20</sup> In certain hypertensive subjects plasma endothelin levels are elevated,<sup>21-23</sup> although other studies did not confirm this.<sup>24-26</sup> However, circulating endothelin levels do not reflect local vascular

production because endothelin is mainly released abluminally.<sup>27</sup>

Endothelin antagonists allow investigation of the role of endogenous endothelin and may provide a new therapeutic approach for hypertension and/or its complications. PD 147953 (or FR 139317)<sup>28,29</sup> is a selective ETA receptor antagonist, whereas PD 145065<sup>30</sup> is a nonselective endothelin antagonist. Both antagonists have been studied in experimental models<sup>22,28-32</sup> but not in humans.

The human skin microcirculation can be investigated noninvasively using a laser Doppler flowmeter.<sup>33-37</sup> This allows testing of new pharmacological concepts with minimal amounts of drugs. We modified the technique for simultaneous application of two drugs without systemic effects. In this study this model has been applied to investigate the effects of endothelin-1 and endothelin-3 and the endothelin antagonists in the human skin microvasculature.

### Methods

#### Study Population

Experiments were performed in male healthy volunteers (age, 22 to 36 years;  $n = 11$ ) who were invited repeatedly to investigate different drugs in the same individual. The subjects were in the supine position during the experiment, with the forearm fixed to avoid artifacts. The study was approved by the Ethics Committee of the Inselspital, Bern, Switzerland. All subjects gave informed consent.

#### Experimental Setup

Room temperature was kept between 19°C and 22°C. Stability of skin blood flow during the experiment was confirmed

Received October 26, 1993; accepted in revised form January 12, 1994.

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at a site where no injection was made in all subjects studied. After a resting period of 20 minutes, the injection site and the measurement point at a distance of 8 mm were marked with a template on the volar forearm. Only four injection sites were allowed per forearm. After baseline measurements, agents were injected with 0.4-mm needles (Omnikan 30, B. Braun). Two injections were performed at the same site within 20 seconds (injection volume, 10  $\mu$ L each). Two substances were injected: as first substance either one of the endothelin antagonists (PD 147953 [ $10^{-11}$  to  $10^{-8}$  mol] or PD 145065 [ $10^{-8}$  mol]) or lidocaine; as second substance, endothelin-1, endothelin-3, or norepinephrine. Control experiments were performed in which saline was injected twice as well as albumin solution at a separate site of the forearm. Injections were made strictly intradermally, producing a symmetrical wheal without visible spreading outside the wheal. If this condition was not fulfilled, the injection site was excluded. Immediately after injection, the investigator marked the third measurement point at 1 mm from the edge of the injection wheal that resulted from the injection of the fluid.

As described,<sup>38</sup> intradermal injection of saline led to a defined and reproducible increase in blood flow within the injection wheal (see "Results") at a distance of 1 mm; at a distance of 8 mm, blood flow did not increase. To exclude nonspecific changes in blood flow due to the peptide structure of the drugs that were used, control experiments with albumin solution were also performed. The vasodilation in response to the albumin solution did not differ from that to saline and even tended to be less pronounced between 4 and 10 minutes after injection. Therefore, changes in blood flow in response to the test agents were expressed as differences to the effects of saline alone. Excellent reproducibility was confirmed at different days in the same subjects with saline and endothelins ( $n=6$ , data not shown).

In pilot experiments, endothelin-1 ( $10^{-15}$  to  $10^{-10}$  mol) was tested; at  $10^{-12}$  mol, maximal contraction within the wheal was achieved, while the vasodilation outside the wheal<sup>34,39,40</sup> was well marked but did not spread more than 3 cm from the injection site. This concentration of endothelin-1 was therefore used.

Injections of all substances were well tolerated by all subjects. A transient pruritus was seen after some injections of endothelin-1. In one case, a transient hypesthesia at the volar surface of the forearm was noted.

### Measurement of Skin Blood Flow

A PF 3 laser Doppler flowmeter (Perimed) with a probe holder was used to assess skin blood flow.<sup>33,35,36</sup> The resulting voltage output, expressed in arbitrary "perfusion units" (PU), is an index of blood flow.<sup>33</sup> Blood flow was measured before and 2, 6, 8, 10, 15, 20, 25, and 30 minutes after injection at the three measurement points. Before the measurement, the device was calibrated for both zero and standard calibrations and put at high sensitivity ("wide band on").

### Drugs

Endothelin-1 and endothelin-3 (Clinalfa), (R)2-[(R)-2-[[1-(hexahydro-1*H*-azepinyl)] carbonyl] amino-4-methylpentanoyl]-amino-3-[3-(1-methyl-1*H*-indolyl)] propionyl] amino-3-(2-pyridyl)propionic acid (PD 147953 or FR 139317, Parke-Davis), Ac-D-10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptene glycine-Leu-Asp-Ile-Trp·2Na (PD 145065, Parke-Davis), norepinephrine, 0.9% NaCl solution, albumin 0.1%, and lidocaine 2% (all Hospital Pharmacy Inselspital, Bern, Switzerland) were used. Solutions of  $10^{-7}$  mol/L of endothelin-1 and endothelin-3 were prepared, corresponding to  $10^{-12}$  mol/10  $\mu$ L. For the endothelin antagonists, solutions of  $10^{-11}$ ,  $10^{-10}$ ,  $10^{-9}$ , and  $10^{-8}$  mol/10  $\mu$ L were prepared, corresponding to  $10^{-11}$  to  $10^{-8}$  mol of substances supplied to the tissue. Solutions were prepared immediately before use to avoid loss of efficacy.

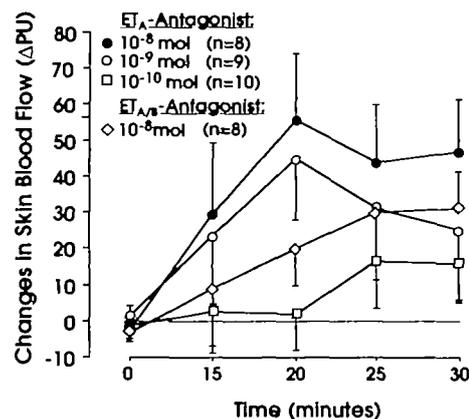


FIG 1. Line graph shows effects of the endothelin-A (ET<sub>A</sub>) antagonist PD 147953 and the endothelin-A/endothelin-B (ET<sub>A/B</sub>) antagonist PD 145065 on skin blood flow at the injection site. Data are shown as mean  $\pm$  SEM. In all experiments the endothelin antagonists and saline were injected. At  $10^{-8}$  mol, the effects of PD 147953 and PD 145065 differed from those with saline ( $P < .05$ ). PU indicates perfusion units.

### Statistical Analysis

The mean between the minimum and the maximum value of a 20-second reading was calculated. The values were registered and analyzed using STAT VIEW 4.01 (ABACUS Inc). Results are expressed as mean  $\pm$  SEM differences between baseline and control; the significance of differences was calculated by multiple ANOVA (95% confidence intervals).

### Results

#### Changes in Skin Blood Flow in the Central Area (Injection Wheal)

##### Saline and Albumin Solutions

The injection of saline, which was used as control, led to an increase in blood flow (maximum increase,  $97 \pm 15$  PU at 6 minutes;  $P < .001$ ;  $n=10$ ); the injection of albumin solution, which was used as peptide control, led to a very similar vasodilation and was even lower than saline between 4 and 10 minutes after injection (maximum increase,  $72 \pm 10$  PU;  $P < .001$ ;  $n=6$ ).

##### Endothelins

Endothelin-1 ( $10^{-12}$  mol) decreased blood flow within the injection wheal (diameter, 4 to 5 mm) compared with control injection (maximum difference from control,  $-79 \pm 14$  PU at 6 minutes;  $P < .001$ ;  $n=11$ ). In contrast, endothelin-3 ( $10^{-12}$  mol) did not affect blood flow within the injection wheal ( $-22 \pm 20$  PU;  $P = \text{NS}$  versus control;  $n=11$ ).

PD 147953 led to a slight dose-dependent increase in blood flow, which reached significance at the highest dose (at  $10^{-8}$  mol,  $+56 \pm 18$  PU at 20 minutes;  $P < .05$ ;  $n=8$ ). The injection of PD 145065 also led to a significant vasodilation ( $10^{-8}$  mol,  $+31 \pm 10$  PU at 30 minutes;  $P < .05$ ;  $n=8$ ; Fig 1).

The endothelin-1-induced decrease in blood flow was fully antagonized by  $10^{-9}$  and  $10^{-8}$  mol of PD 147953 as well as by  $10^{-8}$  mol PD 145065 ( $P < .001$ ). Lower concentrations of PD 147953 ( $10^{-10}$  mol) antagonized endothelin-1 constriction only in part or had no effect ( $10^{-11}$  mol; Fig 2, left panel).

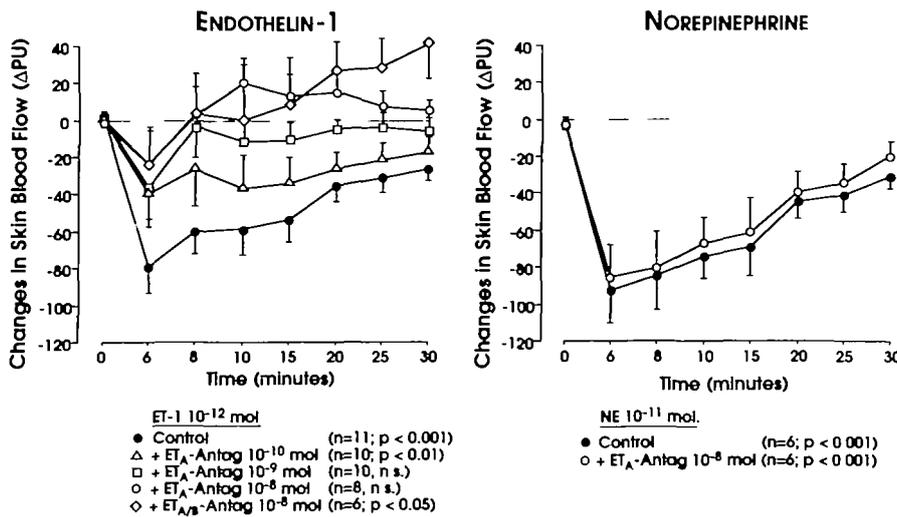


Fig 2. Line graphs show endothelin-1 (ET-1)- and norepinephrine (NE)-induced vasoconstriction in human skin microcirculation. The effects of the endothelin-A (ET<sub>A</sub>) antagonist (Antag) PD 147953 and the endothelin-A/endothelin-B (ET<sub>A/B</sub>) antagonist PD 145065 on ET-1-induced (left) and NE-induced vasoconstriction (right) as measured at the injection site are shown (mean ± SEM). Statistical significance is indicated compared with saline. PU indicates perfusion units; n.s., not significant.

**Norepinephrine**

Norepinephrine (10<sup>-11</sup> mol) decreased blood flow to a degree similar to that of endothelin-1 (maximum difference from control, -93 ± 17 PU at 6 minutes; P < .001; n=6). However, in contrast to endothelin-1, norepinephrine-induced vasoconstriction remained unaffected by PD 147953 (Fig 2, right panel).

**Lidocaine**

Lidocaine, injected alone, led to a slight increase in blood flow (+47 ± 21 PU at 10 minutes; P < .01; n=10). However, the simultaneous injection of lidocaine and endothelin-1 did not affect the decrease in blood flow at the injection site by the peptide (-58 ± 17 PU; P = NS versus endothelin-1; n=10); the simultaneous injection of lidocaine with endothelin-3 tended to increase blood flow (+34 ± 19; P = NS versus endothelin-3; n=10).

**Changes in Skin Blood Flow in the Surrounding Area**

**Saline and Albumin Solutions**

The injection of saline, which was used as control, led to a slight increase in blood flow (maximum increase,

63 ± 9 PU at 6 minutes; P < .001; n=10); the injection of albumin solution, which was used as peptide control, led to a very similar vasodilation (maximum increase, 72 ± 8 PU; P = NS versus saline; n=6).

**Endothelins**

In the surrounding area, endothelin-1 and, to a lesser extent, endothelin-3 (both 10<sup>-12</sup> mol) led to a pronounced vasodilation at a distance of 1 mm (maximum difference from control, +141 ± 27 PU at 6 minutes for endothelin-1 [P < .001]; +96 ± 16 PU at 6 minutes for endothelin-3 [P < .001]) and in particular at a distance of 8 mm from the central area (maximum difference from control, +116 ± 32 PU at 15 minutes for endothelin-1 [P < .001]; +59 ± 16 PU at 6 minutes for endothelin-3 [P < .001]).

The vasodilation caused by endothelin-1 was dose-dependently inhibited by PD 147953. PD 145065 (10<sup>-8</sup> mol) also inhibited this vasodilation (Fig 3, left panel). The vasodilation caused by endothelin-3 was less pronounced but was inhibited dose-dependently by PD 147953 in a manner similar to the inhibition of the vasodilation in response to endothelin-1. PD 145065 (10<sup>-8</sup> mol) also inhibited this vasodilation in response to endothelin-3 (Fig 3, right panel).

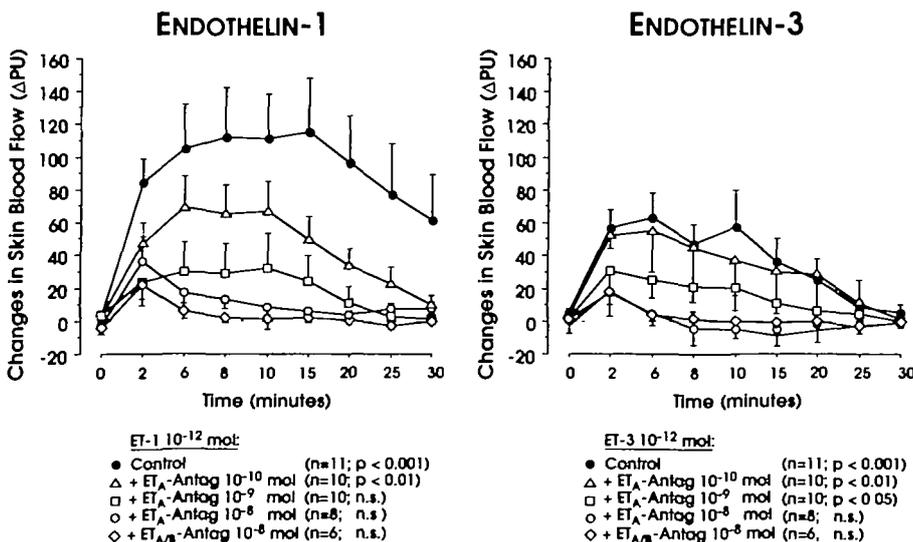


Fig 3. Line graphs show endothelin-1 (ET-1)- and endothelin-3 (ET-3)-induced vasodilation in human skin microcirculation. The effects of the endothelin-A (ET<sub>A</sub>) antagonist PD 147953 and the endothelin-A/endothelin-B (ET<sub>A/B</sub>) antagonist (Antag) PD 145065 on ET-1-induced (left) and ET-3-induced (right) vasodilation as measured at 8 mm from the injection site are shown (mean ± SEM). Statistical significance is indicated compared with saline. PU indicates perfusion units; n.s., not significant.

PD 147953 and PD 145065 injected in the absence of endothelins both increased blood flow at a distance of 1 mm at the highest concentration (maximum increase versus control,  $+48 \pm 16$  PU,  $P < .001$  at 15 minutes [PD 147953,  $10^{-8}$  mol];  $+39 \pm 15$  PU,  $P < .001$  at 10 minutes;  $n=8$  [PD 145065,  $10^{-8}$  mol]). At a distance of 8 mm, PD 147953 as well as PD 145065 had no significant effect on blood flow.

### Norepinephrine

Norepinephrine only slightly decreased blood flow in the surrounding area at distances of 1 and 8 mm compared with control (maximum difference from control,  $-13 \pm 6$  PU;  $P < .05$  at 8-mm distance;  $P < .01$  versus endothelin-1;  $n=6$ ).

### Lidocaine

Lidocaine hydrochloride (2%), injected alone, slightly decreased blood flow (maximum difference from control,  $-33 \pm 12$  PU;  $P < .01$  at 2 minutes at 8-mm distance;  $n=10$ ). The simultaneous injection of lidocaine with endothelin-1 and endothelin-3 ( $10^{-12}$  mol) prevented both endothelin-1- and endothelin-3-induced vasodilation ( $-1.3 \pm 4$  PU,  $P < .01$  versus endothelin-1 alone,  $n=10$ ;  $-2.5 \pm 3$  PU,  $P < .01$  versus endothelin-3 alone,  $n=10$ ).

## Discussion

This study demonstrates that endothelins have profound effects on the human skin microcirculation *in vivo*. Indeed, even low concentrations of local injection of endothelin-1 markedly reduced local blood flow, whereas endothelin-3 was ineffective. Furthermore, both endothelins caused marked vasodilation in an area distant to the injection wheal. The newly developed ETA receptor antagonist PD 147953 and the nonselective endothelin antagonist PD 145065 by themselves caused a slight vasodilation and prevented endothelin-1-induced vasoconstriction in the central area as well as vasodilation in response to endothelin-1 and endothelin-3 distant to the injection wheal.

Endothelins interact with specific receptors that are activated with different potency by the isoforms of the peptide.<sup>4,5,8,13</sup> ETA receptors bind endothelin-1 and, to a lesser degree, endothelin-3; ETB receptors bind endothelin-1 and endothelin-3 with similar affinity.<sup>4,5</sup> ETB receptors on endothelial cells mediate endothelium-dependent vasodilation to endothelins.<sup>9</sup> The fact that endothelin-3 caused no vasodilation in the central area suggests that in the human skin microcirculation endothelial ETB receptors are not expressed or are only sparsely expressed or more likely that the extraluminal application of endothelins does not activate these receptors. The receptors responsible for the vasoconstrictor effects of endothelin, however, seem to be primarily ETA receptors because (1) endothelin-1 but not endothelin-3 caused vasoconstriction in the central area, (2) the specific ETA receptor antagonist PD 147953 prevented endothelin-1-induced contractions in a dose-dependent manner, and (3) the nonselective endothelin antagonist PD 145065 had no additional effect in preventing endothelin-induced vasoconstriction compared with PD 147953. The fact that the vasoconstriction in response to norepinephrine was unaffected by the ETA receptor antagonist demonstrates that its effects were

specific. Because PD 147953 and PD 145065 injected alone caused a slight vasodilation, the local production of endothelin within human skin microvessels appears to contribute to vascular tone. However, because of the limitations of the method applied in the present study, it cannot be excluded that nonspecific reactions contributed to the vasodilation induced by the endothelin antagonists. However, other substances, such as albumin solution or saline, did not lead to a comparable vasodilation. Lidocaine increased blood flow significantly but to a lesser degree than the antagonists, most likely due to the blockade of the local sympathetic nervous system.

Interestingly, around the area of the injection wheal (diameter, 4 to 5 mm), a marked vasodilation occurred with both endothelin-1 and endothelin-3. Because this phenomenon appeared within seconds and distant to the injection wheal, a neurogenic mechanism was suspected. Indeed, as suggested,<sup>34</sup> lidocaine abolished the peripheral vasodilation. Hence, it appears that endothelins not only can affect local vascular tone but can redistribute blood flow to other sites via neurogenic mechanisms. The receptor involved in this response also is likely to be of the ETA type, because PD 147953 inhibited the response dose dependently and PD 145065 had no additional effect. Furthermore, endothelin-3 exhibited a weaker effect than endothelin-1, which further supports the concept that the vasodilation is mediated via ETA receptors, which are preferentially activated by endothelin-1.<sup>4,5</sup> However, the present study cannot fully exclude that ETB receptors also contribute to vasodilation in response to the endothelins. However, this seems unlikely because (1) a selective ETA receptor antagonist would not totally inhibit the effects of ETB-mediated vasodilation, (2) the nonselective endothelin antagonist PD 145065 had no additional inhibitory effects on endothelin-3-induced vasodilation, and (3) other drugs, such as norepinephrine, lidocaine, the endothelin antagonists, albumin solution, or saline, did not induce a comparable vasodilation.

The changes in blood flow that occurred in response to endothelin-1 were substantial. Indeed, in the central area blood flow decreased from baseline levels of 13 to 30 PU to 6 to 8 PU. This is very near "biological zero," ie, the perfusion level measured when arterial flow is occluded by a cuff inflated above systolic blood pressure. In contrast, endothelin-induced vasodilation in the surrounding area averaged 150 to 350 PU. This increase in blood flow was accompanied by a marked reddening of the skin and was more marked than the vasodilation induced by an increase in skin temperature from 30°C to 34°C to 42°C. Hence, the changes in blood flow induced by the endothelins are indeed highly relevant.

In this study we used a laser Doppler flowmetry system to assess vascular responses. With this system changes in local blood flow can be reliably assessed, but it remains a limitation that absolute changes in blood flow cannot be measured. However, the advantage of laser Doppler flowmetry in humans is the fact that it is noninvasive and sensitive enough to record small changes in local blood flow. This has allowed us to test a pharmacological concept in the human microcirculation *in vivo*. Another disadvantage of this experimental setup is the fact that the injection of fluid per se causes vasodilation. However, our pilot experiments and those

of other investigators<sup>38</sup> have demonstrated that this injection artifact is very reproducible even on different days in the same subject. Furthermore, the injection of albumin solution used as peptide control leads to the same vasodilation as with saline. This has forced us to express all effects of hormones and drugs relative to the latter phenomenon. Because of its excellent reproducibility, we believe that this has not altered the results obtained or the conclusions reached. Obviously, this study only assessed responses of skin microvessels, and they may be similar to or different from those of other parts of the circulation. Human subcutaneous resistance vessels obtained with buttock biopsies, however, have been successfully used to elucidate local vascular reactivity in hypertensive subjects.<sup>41</sup>

These results may have important physiological and clinical implications because they suggest that endothelins play a role in local vascular regulation. Although plasma levels in normal subjects are extremely low, the local vascular levels of endothelin appear to exert some vasoconstrictor effects. The new endothelin antagonists<sup>29-32,42,43</sup> are selective and potent tools to study the effects of endogenous endothelin in physiology and disease. Disease states for further clinical studies with endothelin antagonists include systemic and pulmonary hypertension, atherosclerosis, and other vascular diseases as well as acute coronary syndromes and cerebral vasospasm.<sup>19-21,44-46</sup>

In summary, this study in the human skin microcirculation demonstrates pronounced vasoconstrictor effects of endothelin-1 in humans. Furthermore, both endothelin-1 and endothelin-3 cause marked neurogenic vasodilation distant from their site of action. Endothelin receptor antagonists may provide potent and selective tools to further study the biological effects of endothelins in humans in physiology and disease.

### Acknowledgments

This study was supported by the German Research Association (Deutsche Forschungsgemeinschaft, No. WE 1772/1-1), the Roche Research Foundation and the Swiss National Foundation (No. 32-32 655.91 and No. 32-35 591.92 [SCORE]).

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